MUTATIONS IN YEAST METHIONINE AMINOPEPTIDASE-2 INTERFERE WITH BINDING OF THE ANTI-ANGIOGENIC AGENT FUMAGILLIN

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Mutations in Yeast Methionine Aminopeptidase-2 Interfere with Binding of the Anti-angiogenic Agent Fumagillin

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ABSTRACT

The ability of tumors to induce angiogenesis, or new blood vessel formation, is critical to their growth and spread. As a result, factors which inhibit angiogenesis are targets for cancer therapy. One anti-angiogenic agent, TNP 470, is derived from a compound, fumagillin, which induces cell cycle arrest specifically in endothelial cells. Fumagillin has been shown to inhibit the action of a metalloprotease, methionine aminopeptidase-2 (MetAP-2), which is found in both endothelial and non-endothelial cell types. A crystal structure of MetAP-2 with bound fumagillin has recently been solved, which suggests that there are several residues of MetAP-2 in close approximation to fumagillin. These residues may be important in determining specificity of the drug for this enzyme. It was thus hypothesized that by mutating these residues using site-directed mutagenesis, it may be possible to generate mutant versions of MetAP-2 resistant to fumagillin binding. The results presented here demonstrate that three such mutations, G341D, G341S, H339F have been identified. These mutations confer resistance to 10 nM, 5nM, and 1nM concentrations of fumagillin, respectively, which would normally inhibit wild type MetAP-2. If endothelial cells expressing these mutations are resistant to the cell-cycle arrest normally induced by this drug, it constitutes significant functional evidence that the anti-angiogenic effect of this drug can be attributed to its specific interaction with MetAP-2.

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INTRODUCTION

Angiogenesis

Angiogenesis can be defined as growth of new microvessels (1). This process is dependent on the proliferation of capillary endothelial cells. These cells normally divide very slowly, with turnover times on the order of hundreds of days, and therefore define a relatively quiescent tissue (2). However, when stimulated appropriately, these cells can emerge from this quiescent state and undergo brief periods of rapid division. An example of this type of angiogenesis occurs in the ovarian follicle, in which there is a burst of capillary vessel growth which lasts a few days, and is then turned off.

Pathologic angiogenesis occurs when the process of angiogenesis continues to occur for long periods of time, on the order of months to years. An example of this process occurs in tumor growth. In 1974, Folkman hypothesized that in the absence of vascularization, the size and growth of tumors is limited (3). He suggested that there are several "microtumors" developing at any given time in the human body, which never develop into detectable tumors due to lack of adequate supply of oxygen and nutrients. At some point in tumor development, however, these microtumors develop the ability to induce the formation of new blood vessels. These new vessels provide nutrients and oxygen, and act as conduits through which metastases can spread. Although the exact mechanism is unclear, several studies have supported this hypothesis (4). As a result, a new target of cancer therapy has been the development of agents which can selectively inhibit this angiogenic process, thereby returning microtumors to their equilibrium resting state. This is the fundamental goal of anti-angiogenic cancer therapy.

Fumagillin/TNP-470

In 1990, it was shown by D. Ingber that a fungal contaminant, *Aspergillus fumigatus fresenius*, inhibits growth of capillary endothelial cells in culture (5). The active compound secreted by the fungus is fumagillin (Figure 1). A non-toxic synthetic analog of fumagillin, AGM-1470 (Angiogenesis Modulator-1470) has been shown to inhibit capillary endothelial cell growth at picomolar concentrations *in vitro* (6). The clinical preparation is synthesized by Takeda Pharmaceutical Company and called Takeda Neoplastic Product-470, or TNP-470. TNP-470 is a more potent inhibitor of capillary endothelial cell division than fumagillin and has demonstrated little toxicity in studies to date (7-10). Systemic administration of TNP-470 have been shown to inhibit tumor growth in mice, rats, and rabbits, although *in vivo* tumor inhibition requires higher concentrations than *in vitro* cell culture assays (11-14).

The exact mechanism of action of TNP-470 is unknown. However, it has been shown to specifically induce arrest of endothelial cells in the late G_1 phase of the cell cycle (15). TNP-470 results in the inhibition of the retinoblastoma gene product phosphorylation as well as inhibition of cyclin dependent kinases cdk2/4 and expression of cyclins E and A (15). It is known that the late G_1 arrest is not a result of perturbation of early signaling events, since addition of the agent 3 hours after stimulation with growth factor still results in full arrest (15).

Initial explorations into the mechanism of action of TNP-470 revealed that the agent binds covalently to an enzyme, Methionine Aminopeptidase-2 (MetAP-2) , which was initially identified biochemically based on its ability to cleave the amino-terminal methionine residue from peptide substrates (16). This enzyme is found in both endothelial and non-endothelial cells (17),

although the inhibition of the cell cycle has been shown to exhibit specificity to endothelial cell types for unknown reasons (18).

Methionine Aminopeptidases

Members of the methionine aminopeptidase (MetAP) family are cobalt dependent metalloproteases, which function in the selective cleavage of methionine from the amino terminus of peptides and proteins in a non-processive manner (19). Based on sequences comparisons, it has been proposed that two distinct MetAP families exist, MetAP-1 and MetAP-2 (20). Studies in isogenic yeast strains differing only in the presence or absence of functional MetAP-1 or MetAP-2 have shown that fumagillin binds selectively to, and specifically and irreversibly inhibits, MetAP-2 *in vivo* (16).

There is evidence that MetAP-2 serves a critical role in cell growth in addition to its housekeeping function. Experiments in *S. cerevisiae* have shown that deletion of either MetAP-1 or MetAP-2 results in viable, slow-growing yeast. Deletion of both genes results in a lethal phenotype (21). MetAP-2 expression has been shown to correlate with cell growth. In quiescent cells, MetAP-2 levels are below the level of detection using standard immunologic methods. Upon addition of a mitogen, the protein levels increase greatly (22). MetAP-2 has also been identified as a 67kDa protein associated with cell cycle factor eIF-2, and partially reverses protein synthesis inhibition mediated by phosphorylation of eIF-2 *in vivo* (23). It is unlikely that TNP-470 cell cycle arrest is due to interruption of this interaction, because the general protein synthesis inhibition that would result is not compatible with the 7-fold induction in E-selectin protein levels that is seen upon TNP-470 addition (24).

Crystal Structure of Fumagillin-bound MetAP-2

Although the binding to and inhibition of MetAP-2 by fumagillin is well documented, this does not constitute satisfactory evidence that the anti-angiogenic property of fumagillin is due to this interaction. In order to further define the interaction, a 1.8 angstrom resolution crystal structure has recently been solved for human MetAP-2 alone and bound to fumagillin (25). The crystallographic data has shown that human MetAP-2 has a central β sheet with an active site located approximately at the center of its concave face. Two pairs of α-helices and a short COOH-terminal tail cover the sheet's concave face (Figure 2). The active site is a deep pocket with two cobalt molecules at its base, and another pocket that presumably serves as the specificity pocket for the amino-terminal methionine side chain of neutral substrates. Reorientation of the Tyr444 side chain and some water molecules opens this pocket to solvent. This Tyr444 residue is completely conserved in the MetAP-2 family across species.

Molecular Interactions Between MetAP-2 and Fumagillin

The irreversible inhibition of MetAP-2 by fumagillin is the result of a covalent bond formed by an imidazole nitrogen atom of His231 of MetAP-2, and the carbon of the spirocyclic epoxide of fumagillin (16). His231 is believed to be important for the catalytic activity of the enzyme, as an electrophile in an acid-base catalytic triad. The residue does not move significantly with this bond formation, as its nucleophilic imidazole is perfectly positioned to bond with the methylene of the epoxide. The only molecule that moves upon fumagillin binding of MetAP-2 is His339, which rotates its side chain to avoid close contacts with fumagillin (Figure 3). These amino acid residues are therefore critical to fumagillin binding. In addition, several residues are

identified by this crystal structure which are in close approximation to bound fumagillin. These residues include Tyr444, Phe219, Ile338, and His331 (Figure 4). These residues form hydrophobic interactions with the molecule in the deep pocket near the active site of the enzyme. Additionally, the long unsaturated side chain of fumagillin is directed out of the binding pocket and makes two hydrophobic contacts with Leu328 and Leu447, both of which are conserved within the MetAP-2 family (Figure 5) (25).

Relevance of Crystallographic Data

The crystal structure of MetAP-2 bound to fumagillin has allowed careful analysis of the active site and the deep pocket located near the active site. Using this information, it may be possible to identify those residues which are essential in creating a localized environment in the enzyme which is amenable to the binding of fumagillin. Using a rational approach based on these data, an attempt may be made to alter these putatively critical residues of MetAP-2 in order to decrease the affinity with which fumagillin binds the enzyme. MetAP-2 mutations made using this approach may be useful as an initial step in the clarification of the exact mechanism of fumagillin induced cell cycle arrest in endothelial cells. Specifically, MetAP-2 mutations that allow cell viability but have reduced affinity for fumagillin may be tested for the response to a known inhibitory concentration of fumagillin in an endothelial cell system. Demonstration of a reduced potential of fumagillin to induce cell cycle arrest would suggest that MetAP-2 is a critical point in the molecular anti-angiogenic mechanism of the drug. Further clarification of the role of MetAP-2 in the cell may then allow generation of more potent anti-angiogenic compounds which may prove clinically useful.

Summary

Fumagillin and its derivatives are potent anti-angiogenic compounds. It has been shown that these molecules bind and inhibit an enzyme Methionine Aminopeptidase-2, a cobalt metalloprotease. There are two members of the Methionine Aminopeptidase family, MetAP-1 and MetAP-2 that exist in both endothelial and non-endothelial cell types. Fumagillin has been shown to bind and specifically inhibit MetAP-2. MetAP-2 is thought to be responsible for the cleavage of the amino-terminal methionine of proteins and peptides in a non-processive manner. However, in addition to this housekeeping role, MetAP-2 plays an ill-defined, yet critical role in the progression of the cell cycle; cells require either MetAP-1 or MetAP-2 for survival. It is unclear how the functions of MetAP-2 are related. A crystal structure of fumagillin bound to MetAP-2 has allowed a rational analysis of the molecular interactions that the molecule makes with the enzyme. The objective of this study is to determine whether the affinity for fumagillin binding to MetAP-2 can be decreased or eliminated by manipulation of these residues, without destroying the catalytic activity of the enzyme. By demonstrating that mutations can be made in MetAP-2 which decrease the affinity of fumagillin binding, a system can be developed in endothelial cells to test whether these mutations affect the anti-angiogenic properties of the drug. If these properties are indeed altered by mutations in MetAP-2, it constitutes significant evidence that the binding of fumagillin to MetAP-2 is a critical point in the molecular mechanism of the molecule's anti-angiogenic property. Thus this initial study may provide a starting point from which future detailed studies into the molecular mechanism of fumagillin action may be attempted. A detailed knowledge of the anti-angiogenic mechanism

may provide a framework generation of more potent, and highly clinically relevant anti-angiogenic agents.

PURPOSE OF STUDY AND HYPOTHESIS

The purpose of this study is to identify mutations in the MetAP-2 gene that interfere with the binding of the anti-angiogenic agent fumagillin to this enzyme. Given that yeast require either MetAP-1 or MetAP-2 for growth, a yeast null Met-AP-1 strain (ΔMetAP-1) relies on functional MetAP-2 for growth. Fumagillin binds to and completely inhibits MetAP-2. Thus, a *mutation that interferes with the binding of fumagillin to MetAP-2* is any mutation in MetAP-2 that allows a ΔMetAP-1 yeast strain to grow in an inhibitory concentration of fumagillin. That is, a mutation in MetAP-2 that rescues ΔMetAP-1 yeast from growth arrest when exposed to an inhibitory concentration of fumagillin must interfere with the binding of fumagillin to MetAP-2.

The null hypothesis is that the affinity with which fumagillin binds to MetAP-2 cannot be decreased by identifying and mutating critical residues in the MetAP-2 enzyme using a rational approach.

In order to test this hypothesis, the following specific aims are completed:

1. Identification of mutations which are likely to interfere with binding
2. Generation of the chosen mutations
3. Incorporation of mutation into yeast strain
4. Testing mutant yeast strain for ability to grow in an inhibitory concentration of fumagillin.

Given the design of this study, the null hypothesis can be rejected by the identification of a mutation in MetAP-2 which allows yeast growth in the presence of fumagillin at an otherwise inhibitory concentration.

MATERIALS AND METHODS

All experiments described below, including production of all reagents, were designed and performed by the author.

Molecular modeling and selection of desired mutations

Potential mutations were chosen by careful analysis of the crystal structure of fumagillin bound to MetAP-2. These potential mutations were modeled in MetAP-2 on a Silicon Graphics workstation running the O Graphics Molecular Modeling Program. The computer model was studied to predict the effect of the mutation on fumagillin binding. Mutations which appeared to cause steric hindrance thereby making fumagillin binding more difficult were chosen for use in the experiment. There is, however, currently no method of accurately predicting the impact of a given mutation in a protein to a binding partner. Thus, the information gleaned from the molecular modeling described here was simply used as a guideline for mutation selection, along with the crystal structure of the drug-enzyme complex.

Site directed mutagenesis

Mutations were made in the MetAP-2 enzyme by using site-directed mutagenesis, as described in the Stratagene QuickChange Site-Directed Mutagenesis Kit (Figure 6). This method involves the following steps:

1. Preparation and synthesis of PCR Primers and wild type template
2. PCR generation of Mutants
3. Isolation of mutation
4. Confirmation of mutation

1. PCR Primers and wild type template

Primers containing the desired mutation flanked by wild type sequence were synthesized by the Keck Oligonucleotide Facility at Yale University. Primers were designed using guidelines of 25-40mers with >50% GC content, and a melting temperature, (T_m) >65°C (26). Desired mutations were located as close to the center of the primer as possible. Primers were synthesized in both forward and reverse orientations to allow annealing to either strand of the template DNA (Figure 7). In cases where multiple codon choices were available for a single amino acid given the degeneracy of the genetic code, the codon which is most frequently found in yeast to code for the intended amino acid was chosen, as reported in Current Protocols in Molecular Biology. The template plasmid pSE319 is a high-copy episomal plasmid which contains the entire wild type yeast MAP-2 sequence in addition to a replication origin, a trp gene, allowing yeast transformed with this plasmid to grow on tryptophan dropout plates, and an amp resistance gene, allowing for selection on ampicillin plates. Template DNA was isolated from archived bacteria using Qiagen QiaSpin Miniprep Kits.

2. PCR Generation of mutant

In order to achieve optimal ratios of template to primer, each PCR reaction was carried out using three different quantities of template: 6 ng, 12 ng, 18 ng. Template was added to a reaction mix containing: 10mM dNTP, PCR reaction buffer (Stratagene), 125ng forward primer, 125 ng reverse primer, H2O for a final volume of 50 µL. 1U PFU Turbo (Stratagene) was added to each tube. PCR was carried out in a MJ Research PCR machine using the following program:

Step 1.	95°C	30 sec	1 cycle
Step 2.	95°C	30 sec	16 cycles
	55°C	60 sec	
Step 3.	68°C	15 minutes	1 cycle

3. Isolation of mutant DNA

Newly synthesized mutant DNA was isolated using the restriction endonuclease Dpn I (New England BioLabs). This enzyme is a frequent cutting restriction endonuclease which is highly specific for methylated DNA. This allows the enzyme to preferentially cut the methylated template DNA, while sparing the newly synthesized, unmethylated mutant gene. Restriction endonuclease reactions were performed by adding 1U of Dpn I directly to PCR reaction tube, gently mixing, and incubating for 1 hour at 37°C.

4. Confirmation of mutation

After digestion, 5 mL of sample was drawn from each reaction tube and loaded on a 0.9% agarose gel containing ethidium bromide. A standard 1kb marker was used. Wild type pSE319 was used as a positive control. DNA from the positive reactions was sent to the Keck Oligonucleotide Facility at Yale University for sequencing and confirmation of incorporation of the desired mutation. Plasmids which were identified as carrying the desired mutation were used to transform a competent bacterial cell line (DH5α), using the following standard bacterial transformation protocol to provide a renewable source of plasmid.

Bacterial Transformation Protocol
Defrost frozen host cells in ice for 20 minutes
Add PCR mix into tube and incubate 15 minutes on ice
Heat shock host cells at 42°C for 45 seconds
Return to ice for 20 minutes
Add 1mL LB medium to mix

Incubate at 37°C with shaking for 45-60 minutes
Briefly centrifuge mixture and pour off supernatant
Resuspend pellet in small remaining volume of LB
Plate on LB/carbenicillin (50 mg/mL) plate
Incubate for 16-20 hours at 37°C

Yeast transformation

Yeast require either MetAP-1 or MetAP-2 for viability. In order to exploit this requirement, a yeast null MetAP 1 strain (ΔMetAP-1) was used for transformation. This strain is *S. cerevisiae* strain W303 (MAT**a**/MATα ade2-1/ade2-1 his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 ura3-1/ura3-1 trp1-1/trp1-1 can1-100/can1-100) containing *map1::HIS3* disruption. Plasmids containing the desired mutant MetAP-2 gene were used to transform the ΔMetAP-1 yeast strain using the following standard Lithium Acetate yeast transformation protocol. Selection for transformants is accomplished by allowing the yeast to grow on tryptophan dropout plates. Since the vector harbors the tryptophan selection marker, yeast that grow on these plates are confirmed transformants. Single colonies are allowed to grow on these plates and are then selected for the viability assay.

Yeast Lithium Acetate Transformation protocol
Grow ΔMetAP-1 strain in YPD medium for 20-24 hours at 30°C
Use 3mL saturated culture per transformation, transfer to Eppendorf tube
Add 10mL per transformation salmon sperm carrier DNA
Add 10 mL plasmid
Vortex gently to mix
Add 500mL transformation solution (100 mM Lithium Acetate, 40% PEG)
Invert to mix
Incubate at room temperature for 30 minutes
Heat shock at 42°C for 12 minutes
Briefly centrifuge and pour off supernatant
Resuspend pellet in small remaining volume
Plate on tryptophan dropout plate

Incubate 30°C for 4-6 days

Selection of fumagillin resistant transformants

Single colonies were picked from confirmed transformants from standard tryptophan dropout plates. These single colonies were then plated onto tryptophan dropout plates containing various concentrations of fumagillin: 0.2nm, 1nm, 5nm, 10nm. The plates were allowed to incubate at 30°C for 4-7 days and were monitored for growth. Wild type *S. cerevisiae* was used as a positive control on each plate, because it has a functional MetAP-1, which allows it to grow at all the test concentrations of fumagillin used in the study. ΔMetAP-1 yeast strain was used as a negative control on each plate, because it carries wild type MetAP-2, which is fully inhibited at all test concentrations of fumagillin used in the study.

RESULTS

Rational Approach to Mutation Selection

The initial objective in this study was to select a set of mutations in the MetAP-2 enzyme that might have the potential to reduce its binding affinity to fumagillin. This type of prediction can only be speculative; there is no current method for modeling a protein-protein interaction with 100% accuracy. Several amino acid residues in the enzyme were chosen for mutation. These residues are: His339, Phe219, Tyr444, Gly341(Figures 3,5). The His339 residue was chosen for mutation because of the important conformational change that occurs at this residue upon fumagillin binding: the imidazole side change swings out of the way to accommodate fumagillin binding (25). It was thought that replacement of this residue with an amino acid which has a bulky side chain that would not be able to change conformation might hinder fumagillin binding. The mutation H339F was therefore chosen as a study variable. *(All mutations are notated using the standard one-letter amino acid code notation for the wild type amino acid, followed by the position in the protein sequence, followed by the one-letter code for the amino acid that was substituted in the mutant. For examle, H339F indicates that Histidine at position 339 in the wild type MetAP-2 gene was substituted to Phenylalanine in the mutant.)* Additionally, the movement of this histidine imidazole side chain is allowed by the existence of a glycine residue, Gly341. By virtue of lacking a carbon containing side chain, this glycine residue ostensibly provides no additional steric resistance to the movement of the His339 side chain. For this reason, Gly341 was also chosen for mutation to several bulky residues: G341D, G341S, G341V, G341A, G341H.

The deep pocket, thought to be a specificity pocket, situated next to the active site of the enzyme may be important in discriminating the amino terminal methionine of the natural substrates of this enzyme. This pocket contains two residues, Phe219 and Tyr444, which were also chosen for mutation (Figures 3,5). These molecules are in particularly close proximity to fumagillin as demonstrated by the crystal structure, and form the mouth of the specificity pocket. The reactive epoxide of fumagillin, which forms the eventual covalent bond with MetAP-2, is contained in a side chain of fumagillin which protrudes into this specificity pocket. This occlusion of the pocket by mutations in these residues may hinder binding of the molecule as well. Additionally, Tyr444 is conserved in the MetAP-2 family across species, yet an analog is absent in the MetAP-1 family, which may explain some part of the inability of fumagillin to bind MetAP-1 (25). The mutations chosen were F219W, F219I, Y444W, Y444I.

Site-directed mutagenesis

Chosen mutations were made in the desired amino acid positions using site-directed mutagenesis. The PCR products of the desired mutations were run on an agarose electrophoresis gel for evidence of a PCR product. In those experiments that a product was seen, the PCR product was sent for sequencing for confirmation of the desired mutations. Desired sequences of all mutations were confirmed before transformation into ΔMetAP-1 yeast.

Fumagillin Growth Assay

The simplest way to determine whether the binding of fumagillin was functionally decreased as a result of the test mutation was to grow the yeast transformed with the desired mutation on test plates containing varying

concentrations of fumagillin. The concentrations used in this study were 0.2 nM, 1 nM, 5nM, 10 nM. These values were chosen because they represent a range of concentrations which is known to inhibit growth of ΔMetAP-1 yeast strain while remaining non-toxic to wild type yeast. The results are shown in Figure 8. These results demonstrate three mutations, G341D, G341S, and H339F, which are resistant to 10nM, 5nM, and 1nM concentrations of fumagillin, respectively.

DISCUSSION

Methionine Aminopeptidase-2 plays an important yet undefined role in the cell cycle of endothelial cells. Evidence exists which supports MetAP-2 as a binding target of the anti-angiogenic agent fumagillin. It is unclear, however, how this interaction leads to growth arrest specifically in endothelial cells. MetAP-2 is known to have a housekeeping role in the cell which involves cleavage of the amino terminal methionine from many proteins and peptides. Biologically plausible mechanisms exist by which interruption of this function may lead to cell arrest, and evidence that either MetAP-1 or MetAP-2 function is required for cell survival lends additional support to this hypothesis. The N-end rule proposed by Varshavsky, et al. states that the stability of a cellular protein is determined in part by the penultimate amino-terminal residue (27). Thus MetAP-2 may play a role in regulating the stability of proteins. Alteration of the stability of a critical protein which is required for progression through the cell cycle may lead to arrest or growth dysregulation. Furthermore, MetAP-2 plays an important role in protein myristoylation. The covalent attachment of myristic acid to a glycine residue occurs in several important cell signaling proteins, such as certain tyrosine kinase family members, and cyclic AMP-dependent kinase, after the cleavage of the amino terminal methionine by a MetAP (28). Myristoylation has been shown to be required for signaling of several proteins (29). Thus, interruption of MetAP-2 activity may prevent myristoylation of some key signaling protein in endothelial cells which leads to growth arrest.

An alternate explanation for fumagillin activity specifically on endothelial cells is that there is some other interaction in these cells which has not yet been demonstrated that is responsible for growth arrest. Given that MetAP-2 is a housekeeping enzyme which potentially binds many targets

in the cell, it is reasonable to question whether the molecular mechanism for the anti-angiogenic property of fumagillin is indeed related to its binding to this enzyme. The covalent bond formed between fumagillin and MetAP-2 argues against a non-specific binding, but the drug may have some other important consequences in the cell which leads to growth arrest in endothelial cells, independent of its binding to MetAP-2. The objective of this study was to provide a starting point for demonstrating that the anti-angiogenic property of fumagillin is mediated through its binding to MetAP-2. The first step in this process was to determine whether the affinity with which fumagillin binds to MetAP-2 could be decreased by mutation of the enzyme.

The approaches to determining whether MetAP-2 could be mutated to decrease its affinity for fumagillin include: 1. a rational approach, in which the molecular structure of the enzyme is studied for amino acids whose mutation would likely provide resistance for drug binding, or 2. a random approach, in which many mutations are made throughout the enzyme, and the viable mutations are selected and tested. In order to study the binding of fumagillin to MetAP-2, a rational approach was used. The concept of rational drug design involves carefully studying the structure of a molecule, and making only those changes which, based on that structure, have a scientifically plausible reason for attaining the desired goal. In this study, amino acid residues were changed based on either: 1. their proximity to relatively inflexible regions of the fumagillin molecule or 2. their determination of a specificity region of the enzyme as predicted by the crystal structure, with the intention of creating steric hindrances that decreased the affinity of the molecule for the enzyme which could not easily be compensated for by conformational change in the molecule. Likewise selected

residues were mutated to either large, inflexible, or polar amino acids to increase the chances of providing steric hindrance (such as phenylalanine, isoleucine, tryptophan, histidine, aspartic acid, and others).

The benefit of this type of approach is that significant results may be achieved using a relatively small number of mutations, and those results have a plausible biologic explanation for achieving the desired goal. The drawback of this approach is that successful mutations which achieve the desired goal may be missed, because it would have been impossible to predict the effect those mutations would have had.

It was expected at the outset of this project that the binding between MetAP-2 and fumagillin would be impossible to block completely given the highly stable covalent bond that is formed between the reactive epoxide of fumagillin and His231 of MetAP-2. Furthermore, this residue is thought to be critical to the catalytic activity of the enzyme, as an electrophile in an acid-base catalytic triad. Thus, mutation of this residue could potentially destroy the catalytic activity of the enzyme, but the covalent bond formed by this residue makes it unlikely that fumagillin binding can be blocked completely using other mutations in the enzyme. However, a mutation which is found to *decrease* the affinity of fumagillin for MetAP-2, while not completely blocking its binding, would still prove useful in an endothelial cell system designed to study the relationship of fumagillin's binding to MetAP-2 and its anti-angiogenic property. That is, a clear decrease in anti-angiogenic potential of fumagillin associated with a mutation in an endothelial cell MetAP-2 known to decrease the affinity of fumagillin binding would conclusively demonstrate that the drug's mechanism is mediated via its binding to this enzyme.

In order to select potential mutations for testing, the crystal structure of MetAP-2 bound to fumagillin was carefully studied. There are features of the

fumagillin binding site which prompted consideration for mutation selection. The first is the active site of the enzyme. The active site is formed by a deep pocket in the molecule with two cobalt molecules at its base. Additionally, there is a completely covered side pocket that may serve as a specificity pocket for the amino-terminal methionine side chain of the enzyme's natural substrates. This specificity pocket contains many residues which come into close proximity with fumagillin upon its binding to the enzyme. These include, but are not limited to Tyr444, His339, and Phe219 (Figures 3,5). These residues were chosen for mutation based on these close hydrophobic contacts, and the fact that they were not known to be critical for the enzyme function. The mutations chosen were F219I, F219W, Y444I, Y444W.

The second feature of the drug-bound enzyme that was of interest was the residue His339. This is the only residue in the enzyme which undergoes significant motion upon fumagillin binding (25). The imidazole side chain of this residue rotates upon fumagillin binding to avoid close contacts with fumagillin. It was thought that substitution of an amino acid containing a side chain less able to perform this rotation might be a useful mutation. Mutation to phenylalanine was thus attempted. Additionally, there is a glycine residue, Gly341, which is positioned behind His339, which potentially provides space to accept the rotation of the His339 side chain upon fumagillin binding due to its lack of a side chain. It was believed that replacement of this residue with a bulkier residue might also inhibit the rotation of His339 and therefore decrease fumagillin binding affinity. The mutations chosen were thus H339F, G341S, G341V, G341A, G341D, G341H.

In order to generate the chosen mutations in the yeast MetAP-2 gene, a site directed mutagenesis approach was used. In this approach, a

oligonucleotide containing the desired mutation is synthesized, with several bases of wild type sequence flanking it on either side. This oligonucleotide, typically a 40-50mer synthesized in both forward and reverse orientations, is used as a primer for a PCR, using the wild type gene contained in a plasmid as a template. After several successive rounds of PCR, a plasmid containing the desired mutation is generated. The wild type DNA is then digested using a frequent cutting endonuclease which is highly selective for methylated DNA. In this fashion, the newly synthesized, non-methylated DNA is spared, while the template plasmid, which had been harvested from bacterial culture and thus methylated, is destroyed (Figure 6). Representative samples of successful PCR reactions, as demonstrated by the appearance of a band at the expected molecular weight on an agarose gel, were then selected and sequenced for confirmation of incorporation of the correct mutation.

Confirmed mutants were then used to transform a ΔMetAP-1 yeast strain. Use of this strain enabled the simplification of the assay for fumagillin binding. Given that yeast are dependent on either functional MetAP-1 or MetAP-2 for growth, a ΔMetAP-1 strain is dependent entirely on functional MetAP-2. Thus, a simple growth assay can be used to test the effect of the test mutations on fumagillin binding. That is, when MetAP-2 is bound and inhibited by fumagillin, the yeast strain will no longer grow, resulting in a lack of visible colonies on a fumagillin plate. In cases where the mutated MetAP-2 is more resistant to binding than wild type, a higher concentration of fumagillin is required to arrest the yeast strain, resulting in visible colonies on fumagillin plates at concentrations that would inhibit growth of the wild type strain. In the theoretical case in which fumagillin binding is totally blocked by a test mutation, the yeast strain should grow on plates any concentration of fumagillin. The concentrations used for experimentation in

this study were 0.2nM, 1nM, 5nM, and 10nM. These concentrations have been shown previously in the Crews Laboratory to inhibit growth of ΔMetAP-1 yeast containing wild type MetAP-2.

Figure 8 shows the results from the initial test mutations. Seven of the ten attempted mutations displayed no resistance to growth inhibition by fumagillin. It is expected that these mutations which are designed to increase steric hindrance of fumagillin binding may be compensated for by small shifts in the conformation of either the protein or the drug molecule. There is no current means of accurately predicting this type of small conformational shift. Test mutations G341D, G341S and H339F displayed resistance to growth inhibition by fumagillin above wild type at 10nM, 5nM and 1nM, respectively. The decreased potency of fumagillin in these cases can be attributed to decreased affinity of binding due to steric limitations imposed by the test mutation, since this is the only variable changed with respect to wild type. ΔMetAP1 yeast containing wild type MetAP-2 did not grow at 0.2nm, 1nM, 5nM, or 10nM concentration of fumagillin, confirming that these concentrations fall within a range that fully inhibits wild type MetAP-2. The findings presented here confirm the hypothesis that generation of specific mutations in MetAP-2 can interfere with fumagillin binding.

The results presented in this study demonstrate that it is possible to interfere to some extent the binding of fumagillin to MetAP-2. Although it has not been possible in this study to completely block binding of fumagillin to MetAP-2, this may be due in part to the highly stable interaction that is the result of the formation of a covalent bond between the enzyme and the reactive epoxide of the drug. However, the significance of the mutations that have been found to decrease binding affinity is in providing a basis for testing

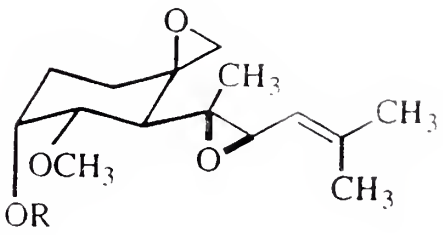
the relationship between the binding of fumagillin to MetAP-2 and its anti-angiogenic potential.

The next experiment that needs to be done to this end is the stable incorporation of the mutations described above into an endothelial cell line. By overexpressing a fumagillin resistant MetAP-2 mutant in an endothelial cell line, a link can be established between the anti-angiogenic property of the drug and its binding to this enzyme. That is, if endothelial cells can be rescued from fumagillin-induced growth arrest by a mutation in MetAP-2 that is known to decrease fumagillin binding, it would be clearly demonstrated that the anti-angiogenic property of fumagillin is mediated through its interaction with the enzyme. This would exclude the possibility that the specific actions of fumagillin on endothelial cells relies on some other function of the drug in endothelial cells that is different that its action in most cells, its interaction with MetAP-2. Initial attempts were made to introduce this mutation into a Bovine Aortic Endothelial Cell (BAEC) line using standard methods, but these attempts have been thus far unsuccessful. Alternate methods are currently under consideration to accomplish this goal.

Other experimentation that could prove useful in the framework of this experiment include the combination of several successful single mutations to generate a set of mutations which together result in increased effectiveness at blocking fumagillin binding. Initial experiments to this end were performed, but none showed any benefit above the single mutations. Other combinations may, however, prove useful. Additionally, the His231 residue of the enzyme can be mutated. This residue is responsible for the formation of the covalent bond between fumagillin and MetAP-2 and is thought to be a critical residue in the catalytic mechanism of the enzyme. Although it is likely that mutation of this residue will result in a non-

functional enzyme, confirmation of this by generation of this mutation would complete this study.

The significance of the data presented in this study lie mainly in that they provide a basis for which future significant research can be conducted. As described previously, the mutations which have been shown to be effective in reducing the binding of fumagillin to MetAP-2 can now be transferred to an endothelial cell system to determine whether the anti-angiogenic effects of the drug are mediated by its interaction with this enzyme. This is a critical piece of information in the delineation of the molecular mechanism of this drug. A clear understanding of the molecular mechanism of this anti-angiogenic agent may allow the generation of more potent, less toxic agents, by identifying new molecular targets. Understanding the molecular basis of this growth pathway in endothelial calls may also provide valuable insights to basic endothelial cell biology, which can have important ramifications to tumor and vascular biology.



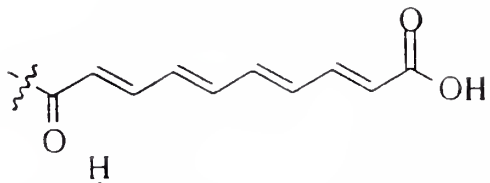
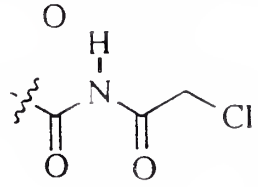
Compound	Structure
Fumagillin	R = 
TNP-470	R = 

Figure 1. Molecular structure of fumagillin. The chemical structure of the anti-angiogenic agent fumagillin is shown, along with its derivative, TNP-470 (16). Reprinted with permission.

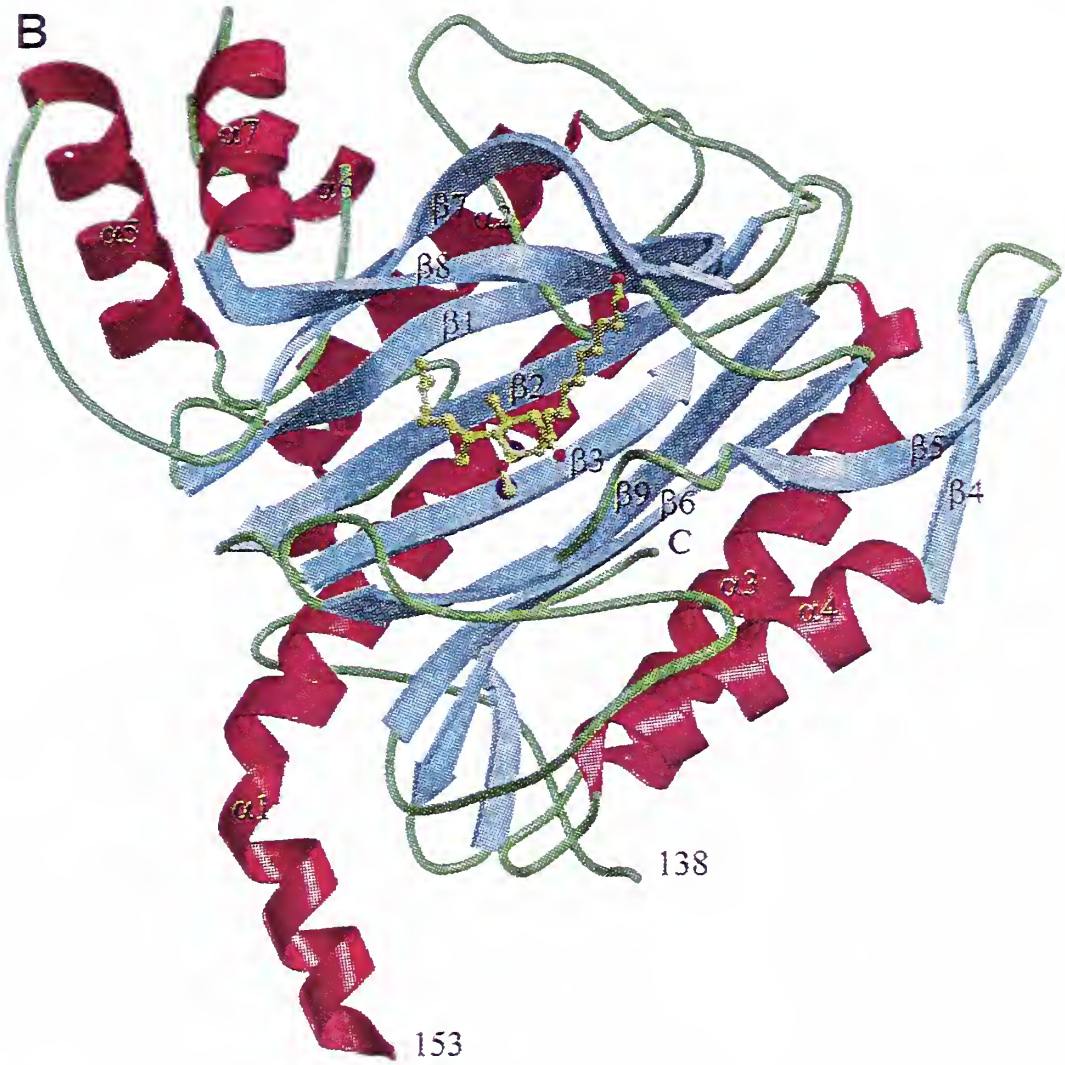


Figure 2. Overall structure of MetAP-2 bound to fumagillin. MetAP-2 (shown in red, green, light blue) is characterized by 7 α helices and 9 β sheets. Fumagillin (shown in yellow and red ball and stick) binds in the active site of the enzyme. The two cobalt molecules (shown in dark blue) are partially obstructed by fumagillin (25). Reprinted with permission.

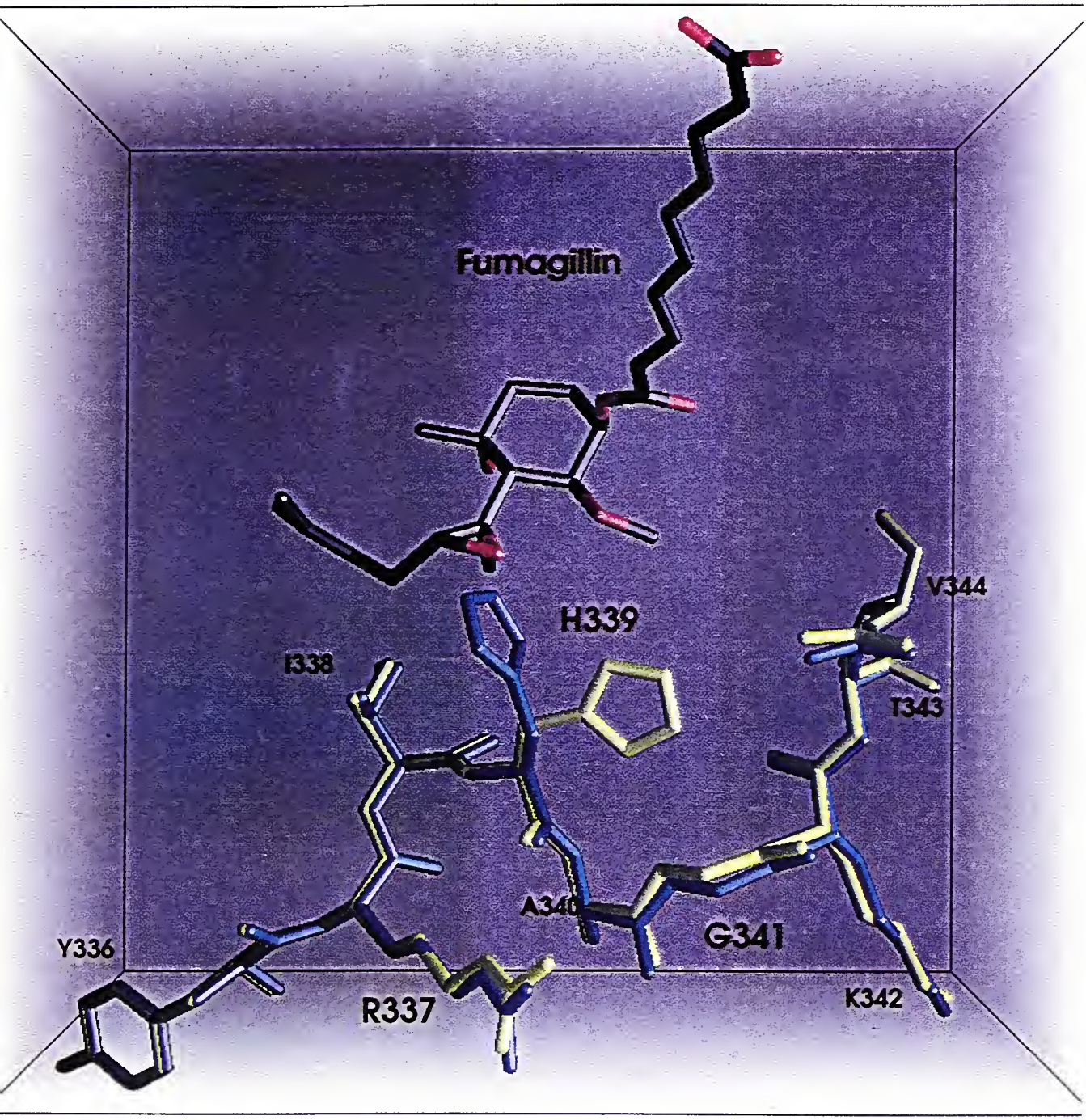


Figure 3. Fumagillin binding causes a conformational change in H339. A portion of the active site of native MetAP-2 is shown in blue. Fumagillin (black and red) bound to MetAP-2 is shown in yellow. A conformational change takes place, shifting the position of the imidazole side chain of His339). Other close contacts are labeled using the one letter amino acid code and a number indicating position in the primary structure. (Figure generated with the assistance of Mark Briedenbach).

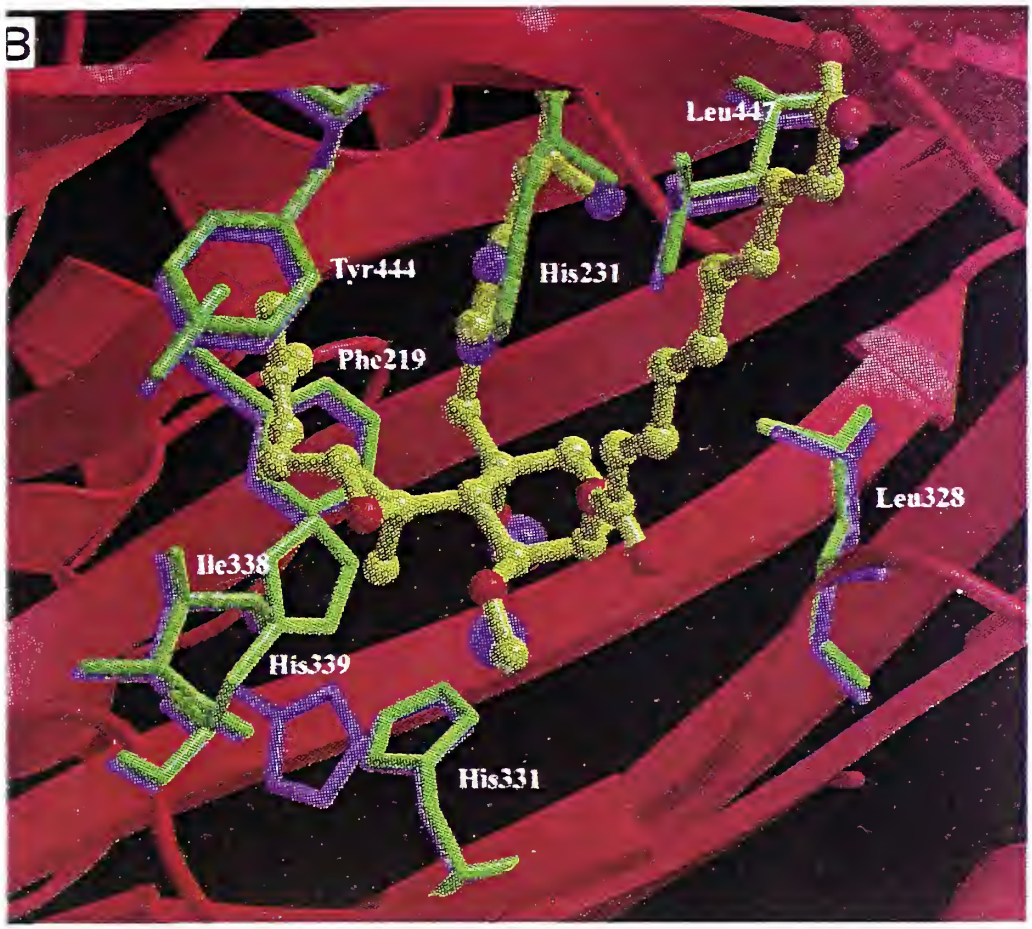


Figure 4. Fumagillin in the active site of MetAP-2. Fumagillin and the covalently attached His231 are shown as ball and stick with carbon yellow, oxygen red, and nitrogen blue. Side chains that interact with fumagillin are drawn as sticks, either in green (native protein) or blue (drug-bound protein) (25). Reprinted with permission.

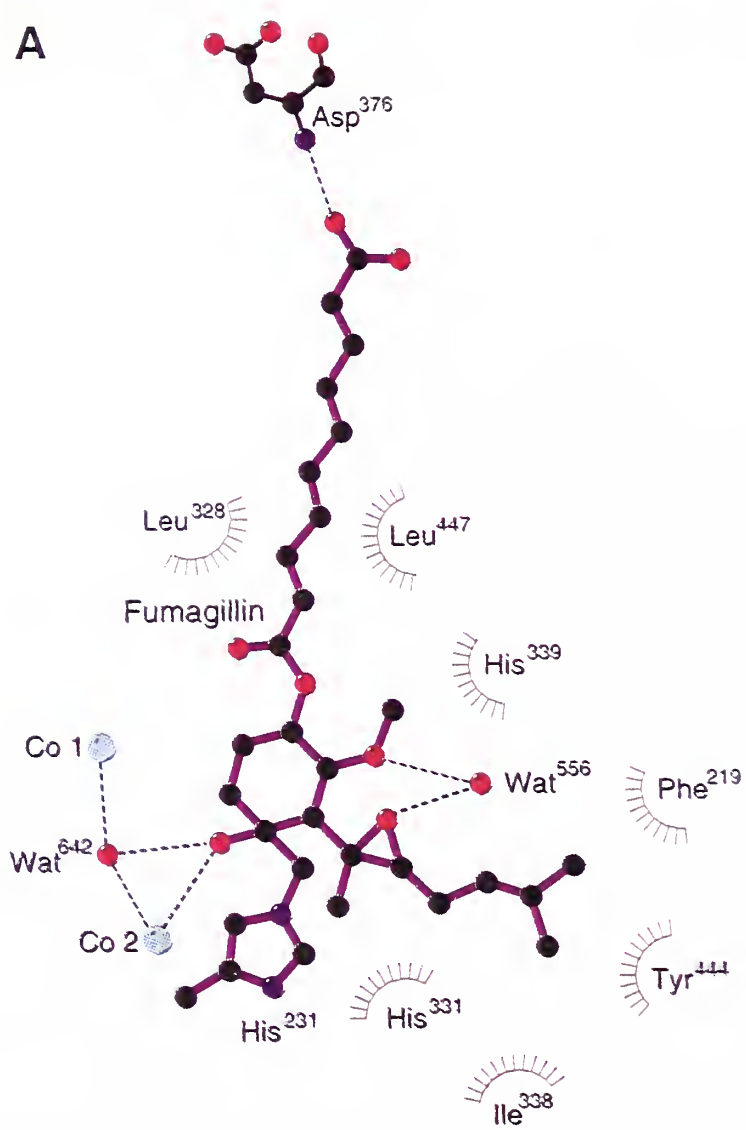
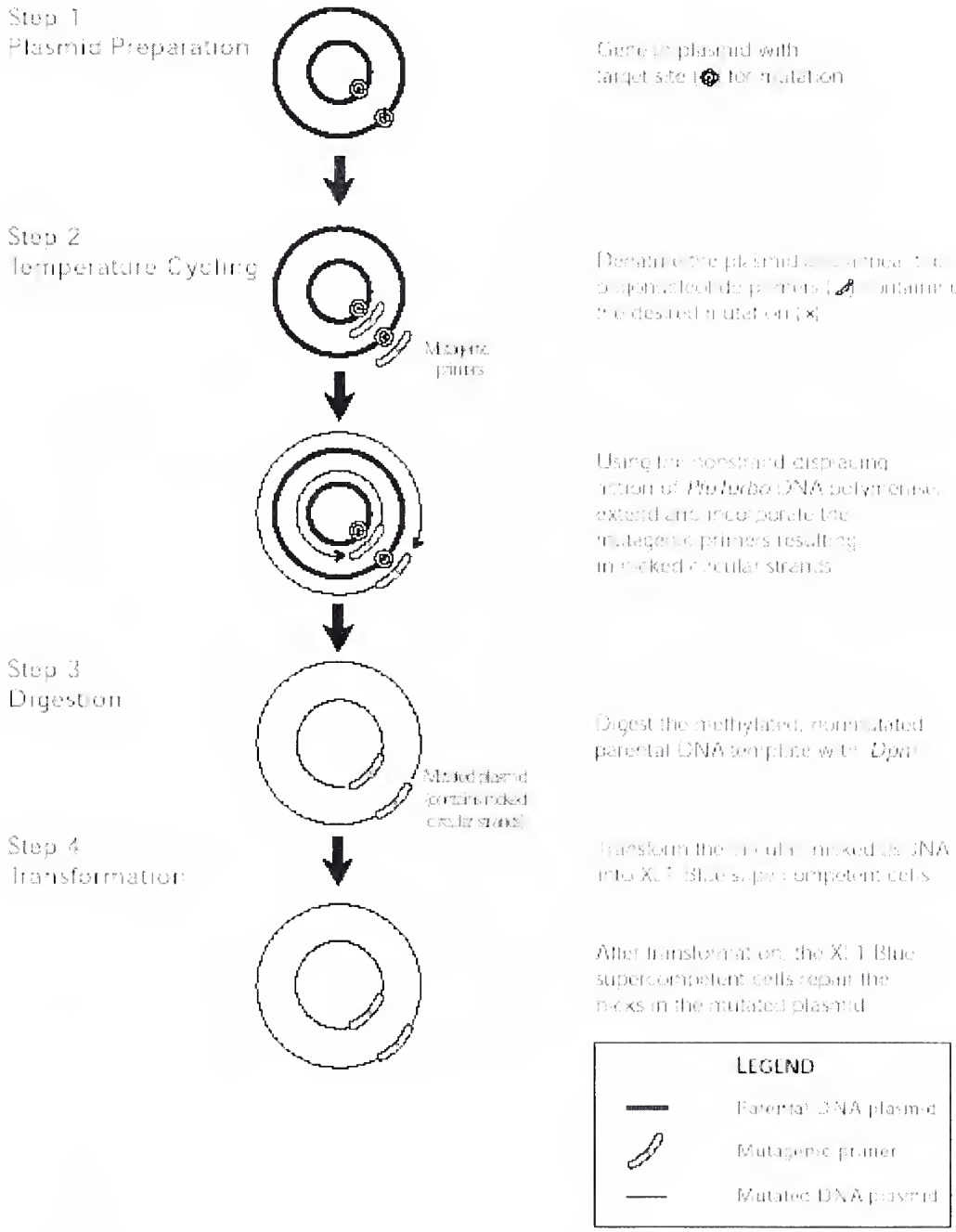


Figure 5. Close side chain contacts to fumagillin in the binding pocket of MetAP-2. Black dashed lines indicate hydrogen bonds, red radial lines indicate hydrophobic contacts. Wat= water molecule, Co= cobalt molecule (25). Reprinted with permission.

Figure 6. Site-Directed Mutagenesis. Template DNA containing the desired target gene is shown. A primer is designed containing the desired mismatch. The PCR reaction is carried out, using the plasmid as a template. The original DNA is digested using Dpn I, leaving only the synthesized DNA containing the desired mutation. DH5-α competent bacteria were used in this study. Figure reproduced from Stratagene QuickChange Site Directed Mutagenesis Manual.



Mutation	Forward (5' to3') Primer
F219I	gga gga tcc caa atc tca agg tat tgg gat tcc aac ggg tct c
F219W	gga gga tcc caa atc tca agg tat tgg gtg gcc aac ggg tct c
Y444I	cgg ttt agt aca gga tat tcc acc att gaa cga tat ccc cgg
Y444W	cgg ttt agt aca gga ttg gcc acc att gaa cga tat ccc cgg
G341D	c gca cca tat cgt atc cac ggc gat aaa tcc gtt ccc
G341S	c gca cca tat cgt atc cac ggc agt aaa tcc gtt ccc
G341V	gca cca tat cgt atc cac ggc gtt aaa tcc gtt ccc atc
G341A	gca cca tat cgt atc cac ggc gct aaa tcc gtt ccc atc
G341H	gca cca tat cgt atc cac ggc cat aaa tcc gtt ccc atc
H339F	gta tcg cac cat atc gta tct tcg gcg gta aat ccg

Figure 7. Primers used in Site Directed Mutagenesis. Forward (5' to 3') sequence of the primer used in site directed mutagenesis are listed. Reverse primers used are the reverse complements of the sequences listed above.

Mutation	Trp-	Fg
F219I	+	0
F219W	+	0
Y444I	+	0
Y444W	+	0
G341D	+	10nM
G341S	+	5nM
G341V	+	0
G341A	+	0
G341H	+	0
H339F	+	1nM

Figure 8. Growth of test mutations on fumagillin. Mutations tested are listed. The second column (Trp-) indicates growth on tryptophan dropout plates. The third column (Fg) indicates the highest concentration of fumagillin on which the strain grew.

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